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ABSTRACT

Biomolecules can be adsorbed on nanoparticles (NP) and degraded during *in vitro* toxicity assays. These artifactual phenomena could lead to misinterpretation of biological activity, such as false-negative results. To avoid possible underestimation of cytokine release after contact between NP and cells, we propose a methodology to account for these artifactual phenomena and lead to accurate measurements. We focused on the pro-inflammatory cytokine tumor necrosis factor TNF-α. We studied well-characterized boehmite engineered NP [aluminum oxide hydroxide, AlO(OH)]. The rate of TNF-α degradation and its adsorption (on boehmite and on the walls of wells) were determined in cell-free conditions by adding a known TNF-α concentration (1500 pg/ml) under various experimental conditions. After a 24-h incubation, we quantified that 7 wt% of the initial TNF-α was degraded over time, 6 wt% adsorbed on the walls of 96-well plates, and 13 wt% adsorbed on the boehmite surface. Finally, boehmite NP were incubated with murine macrophages (RAW 264.7 cell line). The release of TNF-α was assessed for boehmite NP and the experimental data were corrected considering the artifactual phenomena, which accounted for about 20%–30% of the total.

Keywords: boehmite nanoparticles; toxicity; TNF-α adsorption; TNF-α degradation; correction curve

Introduction

The importance of manufactured nanoparticles (NP) has steadily increased for 20 years. In 2015, the share of nanotechnology in the global economy will be about 1000 billion dollars (National Science Foundation, 2003). NP have applications in various fields such as automotive and multimedia industries (Chen et al., 2004; Murphy et al., 2005) and in cosmetics (Nohynek et al. 2007; Somasundaran and Krishnakumar, 1997) as well as in medicine. However, they may have effects on human health by inducing inflammation or oxidative stress. In particular, boehmite NP [aluminum oxide hydroxide, AlO(OH)] are used in a wide range of industrial applications and in many commercial products such as abrasive materials, catalysts, substrates for electronic circuits, and refractory materials; additionally, they are used as vectors in vaccines, replacing conventional adjuvants (Singh et al., 2007). Therefore, due to high potential exposures to boehmite NP, particularly in workplace environments, this type of nanomaterial represents an interesting model for biological activity assessments.

Although some toxicological evaluations have already been carried out, the potential impact on health of boehmite is not yet completely characterized. It has been shown during *in vitro* experiments using RAW 264.7 (murine monocyte/macrophage-like) and A549 (human lung cancer) cell lines that boehmite can induce inflammation (interleukin [IL]-8 production) and cytotoxicity (lactate dehydrogenase [LDH] release) (Kuhlbusch et al., 2009). As a result, boehmite could be considered to carry a potential health risk.

Recent papers clearly show that physicochemical properties should be taken into account in nanotoxicological studies (Boczkowski et al., 2010). Especially for *in vitro* assays, the possible interference between NP and either the medium compounds (assay reagents) or the detection systems (Geys et al., 2010; Horie et al., 2009; Kroll et al., 2009; Monteiro-Riviere et al., 2009) should be carefully considered as well as the role of serum addition on

dispersion and adsorption at the particle surface (Murdock et al., 2008) should be carefully considered. Moreover, some studies also suggest possible biases in the evaluation of the biological toxicity, particularly when the release of cytokines is assessed (Val, 2009). Indeed, the cytokines can be adsorbed at the surface of the NP, resulting in an underestimation of the quantity released (Akhtar et al., 2010; Cedervall et al., 2007; Val, 2009).

Cedervall et al. (2007) introduced an approach that identifies proteins on NP (*N*-isopropylacrylamide-*co*-*N*-*tert*-butylacrylamide) and the exchange with plasma proteins (human serum albumin). Val (2009) measured the capacity of titanium dioxide and carbon black NP to adsorb different pro-inflammatory cytokines, including IL-6, granulocyte monocyte colony-stimulating factor (GM-CSF), and tumor necrosis factor TNF-α. Using different types of particulate matters and placing them in contact with standard IL-8 in a cell-free system, Akhtar et al. (2010) showed that particles exhibited differential IL-8 adsorption capacities depending on their physicochemical properties. These results tend to lead to the same conclusions: protein or cytokine adsorption on NP depends on NP surface characteristics and size; furthermore, the nature of the biomolecules adsorbed on the NP will affect the biological toxicity.

Additionally, Turci et al. (2010) used a new set of complementary techniques to characterize and compare the adsorption of different proteins on silica NP. The proteins studied were bovine serum albumin, hen egg lysozyme, bovine pancreatic ribonuclease A, and bovine lactoperoxidase. Although adsorption was influenced by the net protein charge, the surface charge distribution of proteins determined the affinity for the solid support (silica NP) and the stability of adsorption of proteins on silica NP.

Finally, Xia et al. (2010), using predictive models, described the kinetics of the protein corona formation. They used a biological surface adsorption index (BSAI) to characterize the adsorption properties of NP by quantifying the competitive adsorption of a set of small

molecule probes onto NP by mimicking the molecular interactions of the NP with proteins. By these means, the molecular interactions of NP with proteins could be characterized by the BSAI nanodescriptors, which govern the adsorption affinity and selectivity of biomolecules onto the surfaces of nanomaterials in the corona-formation processes. These results were used to determine if the findings obtained for biological activity were correct or if they represented a false-negative result due to an artifact such as adsorption on NP.

The objectives of the present study were to highlight artifacts that could occur during *in vitro* toxicity assessments, especially in TNF-α measurement, and to propose a methodology to quantify them in order to correct a possible underestimation of the TNF-α release. The TNF-α was chosen because inflammation can potentially be triggered by NP and the assessment of this cytokine is a very common test in nanotoxicology. This study, applied to the TNF-α, rather represents a concept as the developed methodology can be extended to other kinds of molecules. Boehmite NP were incubated either in cell-free conditions or with a murine macrophage cell line (RAW 264.7). The experiments were conducted at two temperatures (4 °C and 37 °C), with two types of support (glass flask or polystyrene 96-well plates). We demonstrated that in addition to adsorption, TNF-α degradation also occurred. We finally proposed a correction applicable to boehmite, taking into account these two types of artifactual phenomena. The methodology for establishing the correction is easily transposable to other kinds of NP and biomolecules.

Material and Methods

Physicochemical characterization of NP

Disperal® boehmite NP were purchased from Sasol (Germany).

The morphology of NP was analyzed using electron microscopy in the scanning electron microscopy (SEM) mode with a field-emission scanning electron microscope (JEOL JSM-6500F) or in the transmission electron microscopy (TEM) mode with « X » transmission electron microscope.

The specific surface areas were determined by N_2 adsorption at 77 K after out-gassing for 2 h at 200 °C (Micromeritics ASAP 2000), using the Brunauer-Emmet-Teller (BET) method.

NP densities were determined using a gas pycnometer (Micrometrics AccuPyc 1330) working under a helium atmosphere. Results are expressed as the means of 10 successive measurements on the same sample. For the zeta potential and size measurement, the boehmite suspension was fixed at 100 mg/L and prepared both in water and in complete Dulbecco's modified Eagle's medium (DMEM). Analyses were performed using a diffusion light scattering (DLS) device (ZetaSizer nano S, Malvern Instrument). X-ray diffraction (Siemens D5000 apparatus) experiments were performed at room temperature to examine the presence of crystalline phases. The crystallite size was calculated from the diffractograms obtained using the Scherrer relation (Topaz-4P software).

In vitro *assays*

Cell line and culture conditions

The RAW 264.7 macrophage-like cell line, derived from mice, was provided by the ATCC Cell Biology Collection (Promochem LGC). Cells were cultured in DMEM (Gibco)

complemented with 10% fetal calf serum (Gibco), 1% penicillin-streptomycin (penicillin 10,000 U/ml, streptomycin 10 mg/ml, Sigma) and incubated at 37 °C under a 5% CO_2 humidified atmosphere (Leclerc et al. 2010).

Boehmite (AlOOH) NP were resuspended in complete DMEM (DMEMc), vortexed for 30 s and then different concentrations were incubated with cells: 150, 300, 600, and 1200 µg/ml. Suspensions were freshly prepared just before experiments and discarded after each test series without any storage. We checked the hydrodynamic size of boehmite particles by DLS.

For each experiment, cells were prepared in 96-well plates (100,000 cells/well) in 25 µl of DMEMc. Seventy-five microliters of each boehmite suspension was added to the culture and then incubated for 24 h at 37 °C in a 5% CO_2 atmosphere.

Three independent experiments were performed using four boehmite NP concentrations (150, 300, 600, and 1200 µg/ml) and were tested for each of the following conditions: cells alone (negative control of toxicity), DQ 12 quartz (positive control of toxicity) (Bruch et al. 2004), and boehmite NP.

LDH release

The activity of the LDH released from cells with damaged membranes was assessed using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's instructions. Detection was performed on a fluorometer (Fluoroskan Ascent, Thermolabsystems), using 530/590 nm excitation/emission wavelengths. The activity of the released LDH was reported in comparison to total cellular LDH (measured after control cell lysis) and was expressed as a percentage of the control.

Pro-inflammatory TNF-α response

After a 24-h incubation with NP, the release of TNF-α was assessed in the culture supernatant using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine® Mouse TNF-α Immunoassay, R&D Systems). The optical density of each well was determined according to the manufacturer's instructions by using a microplate reader (Multiskan RC, Thermolabsystems) set to 450 nm. A standard curve was established and results were expressed in picograms of TNF-α per milliliter of supernatant.

Assessment of artifactual phenomena

TNF-α degradation

The TNF-α standard solution used for the assessment of adsorption and degradation phenomena was provided by the ELISA kit (Quantikine® Mouse TNF-α Immunoassay, R&D Systems). The assays were performed in a cell-free environment. Since adsorption and degradation always occur simultaneously, to study these two phenomena independently glass flasks were used presenting a higher volume/surface ratio than wells of 96-wells plates (190 and 66 µl/cm² respectively). In these conditions, TNF-α adsorption becomes negligible over its degradation. Therefore experiments carried out in glass flasks allowed to limit adsorption and focus on degradation.

A defined amount of TNF-α (1500 pg/ml) was suspended in DMEMc (without NP, in cell-free environment) in a glass flask at different temperatures: 4 °C (refrigerator) and 37 °C (incubator) for different times (3, 16, 24, and 48 h). The 4 °C temperature corresponded to the TNF-α storage condition (to avoid any degradation phenomenon) and 37 °C corresponded to the *in vitro* experimental conditions. The difference between the results at 4 °C and 37 °C permitted to determine the quantity of degraded TNF-α.

TNF-α adsorption on the support walls

A concentration of TNF-α (1500 pg/ml) was added to DMEMc in 96-well plates, at 4 °C and 37 °C for different durations (3, 16, 24, and 48 h). The difference between the results in glass and in plastic allowed to determine the quantity of adsorbed TNF-α on the walls of wells.

TNF-α adsorption on boehmite

A concentration of TNF-α (1500 pg/ml, representing 75 pg of TNF-α per well) was introduced with boehmite (1200 µg, representing 12 µg of NP per well) in DMEMc in 96-well culture plates. After a 24-h contact, supernatants were collected, and the concentration of TNF-α was evaluated as previously described. The mass variation of TNF-α was then calculated.

Adsorption isotherms of TNF-α

Different TNF-α/boehmite weight ratios were established (0.78, 1.56, 3.13, and 6.25) by adding varying amounts of TNF-α to cell cultures. Contact lasted for 1, 3, 16, and 24 h at 37 °C in DMEMc in 96-well culture plates to determine the kinetics of TNF-α adsorption on boehmite. For each condition, the residual TNF-α was measured using the same ELISA procedure as previously described. For each ratio, the mass of adsorbed TNF-α was determined with regard to the duration of contact. It was estimated as the difference between the initial and residual concentrations of TNF-α in the supernatant, taking care to subtract degraded TNF-α as previously determined. When the adsorbed TNF-α reached a maximal level, the curve describing the mass of adsorbed TNF-α, depending on the mass of residual TNF-α, could be drawn. It represented the isotherm curve at the thermodynamic equilibrium, and it allowed correction of the results for the amount of TNF-α degraded and adsorbed on well walls and NP.

Statistical analysis

Analysis and graphics were performed on Prism 5.0 software (GraphPad, San Diego, CA). Significance was established with ANOVA test ($p < 0.05$).

Results

Physicochemical characterization of boehmite NP

Specific surface area, density, electron microscopy, and X-ray diffraction

Boehmite is made of spherical particles. Although some particles exhibit a diameter of about 50 µm, the major part is much smaller (from 1 to 10 µm in diameter). Small elements like platelets of around 20 nm can be observed by SEM (Figure 1).

The specific surface area, S_{BET}, was measured as 157 m^2/g. The density, $\rho_{pp,}$ was determined to be 3.28 g/cm^3 by using the helium pycnometer. Using these data and Equation 1, we determined a primary particle diameter (d_{pp}) of 11.7 nm, assuming a spherical shape of boehmite nanoparticles as confirmed by electron microscopy observation (Figure 1).

$$d_{pp} = \frac{6}{\rho_{pp} \times S_{BET}} \qquad [1]$$

where d_{pp} is expressed in micrometers, ρ_{pp} in grams per cubic centimeters, and S_{BET} in squared meters per gram.

The position of X-ray diffraction peaks was in agreement with the data reported for boehmite crystalline phase (JCPDS n°21-1307). The broad diffraction lines revealed a crystallite size of 10 ± 0.1 nm according to the Scherrer relation (Figure 2). This size was in perfect accordance with electron microscopy data.

Particle size and zeta potential measurement

DLS assays (Figure 3) clearly showed that aggregation of the NP occurred in water and increased with pH. At pH 3.5, a narrow particle-size distribution was observed with a maximum at 50 nm; at pH 6.9 the maximum was located at 315 nm; and finally at pH 9.4, the particle size was 770 nm. In DMEMc (pH 7.4) NP showed a broad particle-size distribution, with a maximum at 825 nm.

Thus, at a similar pH, boehmite NP are more aggregated in DMEMc than in water (825 nm and 315 nm, respectively).

Zeta potential measurement proved that NP have a positive surface charge until pH 9.5 (the isoelectric point); beyond this value, NP are negatively charged. This result explained the rise of agglomeration observed with pH. As a matter of fact, the more the pH was near the isoelectric point, the more the NP were agglomerated.

In vitro *toxicity assessment*

LDH release

Cells incubated for 24 h with boehmite NP released the same level of LDH as the negative control (cells tested alone, without NP) and no dose-dependent effect was observed (Figure 4A).

The positive control of toxicity (DQ12 quartz) exhibited a level of released LDH that was significantly higher and dose dependent (34% of released LDH for 1200 µg/ml of boehmite).

Pro-inflammatory TNF-α response

The amount of TNF-α released by cells alone was quite negligible (250 pg/ml) (Figure 4B). No significant difference from the control group was observed when boehmite was incubated with macrophages for 24h (300 pg/ml of TNF-α produced). However, incubation with the DQ 12 quartz positive control induced a high TNF-α release (800 to 1000 pg/ml) which was clearly dose-dependent.

Adsorption and degradation of TNF-α in cell-free conditions

TNF-α degradation

The experiment in the glass flask at 4°C clearly showed no significant adsorption and no degradation phenomena (Figure 5). For the same experimental conditions at 37°C, we assumed that the observed mass change of TNF-α was due only to the degradation phenomenon (Δm TNF-α of 6.5 wt% after 24 h). Finally, the amount of TNF-α degraded in DMEMc was dependent on the duration of the experiment and the temperature, and we approximated the rate of TNF-α degradation at 0.12 pg/h at 37 °C in DMEMc.

TNF-α adsorption on the walls of the wells

After 24-h contact at 37°C in DMEMc without boehmite, 11.6 wt% loss of TNF-α was observed in polystyrene wells. Under these conditions, both TNF-α degradation and adsorption on the walls of the well occurred. However, loss only reached 6.5 wt% in the glass flask in which only degradation occurred, given the very low surface to volume ratio of the flask. Thus, we demonstrated significant TNF-α adsorption on the walls of wells in which we did the assays. This adsorption was calculated by the difference between experiments in wells and in the glass flask.

TNF-α adsorption on boehmite

A significant increase in TNF-α mass loss (Δm TNF-α) was observed after a 24-h incubation of cells with boehmite (24.8 wt% versus 11.6 wt% without boehmite) (Figure 5). These results suggest that significant TNF-α adsorption on boehmite occurred. A saturation of TNF-α adsorption on boehmite can also be noticed as a plateau was reached (about 25%).

Adsorption isotherms of TNF-α

The kinetics curve of adsorption revealed that the amount of TNF-α adsorbed on the boehmite surface reached a thermodynamic equilibrium at 24 h, regardless of the boehmite to

TNF-α weight ratio (Figure 6). Thus, the curve representing the adsorbed TNF-α based on the residual TNF-α could be drawn (i.e., the adsorption isotherm curve), after subtracting the amount of degraded TNF-α, which was calculated based on the previously determined TNF-α degradation rate of 0.12 pg/h.

Using the adsorption isotherm curve and knowing the amount of residual TNF-α in the supernatant, we could determine the amount of adsorbed TNF-α. For example, for a weight dose of 1200 µg/ml of boehmite in contact with the macrophage cell line, the concentration of TNF-α released after 24 h in the supernatant was about 300 pg/ml (Figure 4); i.e., 1.25 pg of TNF-α in solution per microgram of boehmite initially introduced. The adsorption isotherm (Figure 7) led us to estimate that if there was 1.25 pg of TNF-α in the supernatant per microgram of boehmite, 0.2 pg of TNF-α was adsorbed per microgram of boehmite. Since the mass of boehmite introduced per wells was 12 µg, we calculated that 2.4 pg of TNF-α would be adsorbed on boehmite. Therefore after a 24-h incubation for a weight dose of 1200 µg/ml of boehmite, we estimated 26% of Δm TNF-α artifacts, taking into account adsorption and degradation phenomena.

Corrected data

Figure 8 represents our data after correction of TNF-α adsorption and degradation artifacts as previously determined. TNF-α degradation and adsorption on the walls of wells is constant (about 13%), whereas TNF-α adsorption on boehmite NP must be calculated for each experimental condition as reported on Figure 8.

After correction of the artifacts, the TNF-α production was found to be higher than that of cells unexposed to NP even though the difference was not judged statistically significant (ANOVA test, $p>0.05$).

Discussion

There have been concerns in recent years about the effects of NP on health (Oberdorster et al., 2005). Many studies have described the pro-inflammatory effects of different NP, particularly in the respiratory system, which represents the main route of exposure (Oberdorster et al., 2005; Singh et al., 2007; Xia et al., 2006).

The binding of proteins to NP surfaces is a well-known phenomenon that is specific to the NP surface characteristics and also depends on the nature of the proteins. For instance, adsorption of TNF-α on carbon black and titanium dioxide NP has been reported, and the adsorption of other cytokines such as IL-6 and GM-CSF has also been observed (Val, 2009). Models describing the kinetics of the adsorption of the proteins on NP have been established (Dell'Orco et al., 2010; Xia et al., 2010). The main objective of the present work was to provide new insight on the possible artifacts of measures that could occur during *in vitro* toxicity assessments. We focused our attention on TNF-α, evaluating its adsorption and degradation. We aimed to demonstrate that a correction could be established to avoid underestimating cytokine release in the presence of nanoparticles that might have a high adsorption capacity due to their huge surface area. Owing to a lack of data in the literature about TNF-α adsorption on nanoparticles, we selected boehmite to illustrate that the potential impact of artifacts associated with nanoparticles is not negligible. To that purpose, *in vitro* TNF-α measurements were performed in a cell-free environment or after contact with a macrophage cell line.

First, different techniques were used for physicochemical characterization to evaluate the size of primary particles. All techniques yielded the same conclusions: boehmite nanoparticle size is about 10 nm, and the particles have a strong tendency to aggregate in suspension. The DLS technique was used to assess the state of NP aggregation in cell culture medium and in water, and the observations of different sized NP aggregates in cell culture

medium were in agreement with literature reports (Limbach et al., 2005; Singh et al., 2007; Xia et al., 2006). However, the size of aggregates depended on the pH of the solution as measured by variations of the zeta potential (Turci et al., 2010). For boehmite NP, aggregation increased with the pH.

Secondly, the pro-inflammatory effect of boehmite NP was evaluated after 24-h contact with macrophages. In a global manner, TNF-α production was limited (about 300 pg/ml) compared with the positive control for toxicity (800–1000 pg/ml).

In order to check the accuracy of our results and eliminate potential experimental biases, we investigated whether TNF-α degraded over time and whether it could be adsorbed at the surface of the NP and on the support. Indeed, adsorption caused a strong underestimation of the amount of TNF-α release as quantified by ELISA. To that purpose, we introduced a defined amount of TNF-α (1500 pg/ml) in solution, in cell-free conditions with and without boehmite NP, and we quantified the amount of degraded and/or adsorbed TNF-α. We were able to establish the following relationship:

$$[\text{TNF-}\alpha]_{\text{in the supernatant}} = [\text{TNF-}\alpha]_{\text{produced by cells}} - [\text{TNF-}\alpha]_{\text{adsorbed on (NP+wells)}} - [\text{TNF-}\alpha]_{\text{degraded}} \quad [2]$$

Each term of this equation was studied, and the disappearance of TNF-α over time and as a factor of temperature was observed. When 75 pg of TNF-α was added to 12 µg of NP in cell culture medium, the mass of TNF-α measured after 24 h at 37 °C was only 58 pg, representing a loss of 26 wt%. We specifically established that 7 wt% TNF-α was degraded, 6 wt% TNF-α was adsorbed on walls of wells, and 13 wt% TNF-α was adsorbed on the boehmite NP surface.

However, results may depend on the biomolecules and the type of NP studied. For instance, Val (2009) showed that IL-6 was not adsorbed on carbon black and titanium dioxide, but in contrast GM-CSF and TNF-α were completely adsorbed by carbon black and partially by titanium dioxide (60% for GM-CSF and 26% for TNF-α). Our results are in agreement with this study. We observed an adsorption of TNF-α on boehmite NP and on the support of the assays, but we also demonstrated disappearance of TNF-α due to degradation.

The interactions between LDH and NP have not been investigated in this study as it was beyond the scope of the present paper. The aim of this work was to highlight the fact that artifacts can occur in common nanotoxicological tests and to avoid misinterpretation of the results a methodology could be developed to take them into consideration and correct them. We did not mean to be exhaustive and we chose to illustrate this concept with a very frequently assessed cytokine: TNF-α. Of course, this concept can be extended to other molecules but generalities cannot be drawn, the corrections to bring are highly dependent on the nature of the NP, the materials, the protocols… as already mentioned (Val, 2009). Actually, it is very likely that the same phenomenon as described for TNF-α occur for LDH and these artifacts must be measured too for a comprehensive study. Furthermore, artifacts in the LDH assessment could explain the slight decrease in the amount of released LDH by cells exposed to boehmite NP compared to that of control, unexposed cells. However, assessing LDH artifacts is currently impossible because of technical limitations. We were able to study TNF-α artifacts because a standard TNF-α solution was provided in the detection kit. Therefore by adding a known amount of TNF-α in various experimental conditions we could easily follow a variation of its mass and deduce from this Δm the amount of degradation and/or adsorption of this molecule. This method is clearly impossible to transpose to the LDH assay as no LDH standard solution is provided in the detection kit (which would be the best standard) nor is commercially available. We can find LDH extracted and purified from human

or different species but to the best of our knowledge no murine LDH was found. And it will be a non sense to compare things which are from different species and therefore are not comparable. The possibility to recover LDH from lyzed cells was also considered but in this case cell lysats would also contain various biomolecules which would interfere with the kit reagents and induce other artifacts in the measures.

Keeping in mind the models describing the kinetics of the adsorption of the proteins on NP (Dell'Orco et al., 2010; Xia et al., 2010), we performed a kinetic study. Results allowed us to plot a corrective curve when the thermodynamic equilibrium was reached for different NP to TNF-α, ratios which led us to establish a correction to avoid misinterpretation of TNF-α release in the presence of nanoparticles.

After such a quantitative evaluation of the artifacts the original data were corrected to take into account these biases and give more accurate results.

Biomolecule adsorption onto material surface (e.g., nanoparticles) occurs by multiple short-range interactions (H-bond, electrostatic, van der Waals or dispersive forces, hydrophobic effect) that are individually rather weak (a few k_bT or less), but additively they induce a significant adsorption energy. The classic view of Langmuir isotherms assumes that some interaction energy is reversibly available to create a biomolecule–material surface complex. The amount of adsorbed biomolecules is limited by the available area on a material's surface, thus the surface area of nanoparticles is doubtless a key parameter influencing the adsorption artifact in *in vitro* nanotoxicology experiments. Moreover, when the NP surface is functionalized or covered with a given biomolecule, its surface properties are changed, and this modifies the adsorption of other biomolecules remaining in solution. As a result, nonspecific adsorption of proteins such as bovine serum albumin or casein can be proposed to sterically "block" the material surface so as to prevent the unwanted adsorption of valuable and interesting biomolecule such as cytokines. Nevertheless, as the physicochemical

features of a surface compose a key point to be taken into account in nanotoxicology studies, the saturation of nanoparticles' surfaces may strongly modify the biological activity compared to uncoated nanoparticles.

In conclusion, we have studied the physicochemical characteristics of boehmite NP and their capacity to adsorb a specific biomolecule, TNF-α, which is often assessed in *in vitro* toxicological studies. Results showed the existence of an adsorption process conjugated with degradation, leading to underestimation of the actual amount of TNF-α released during *in vitro* toxicity assays. To our knowledge, this is the first study introducing a correction that takes artifacts into account and is adaptable to other types of biomolecules and NP.

Conflict of interest statement

The authors report no conflicts of interest.

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FIGURES

Fig. 1. (A) SEM and (B) TEM images of particles.

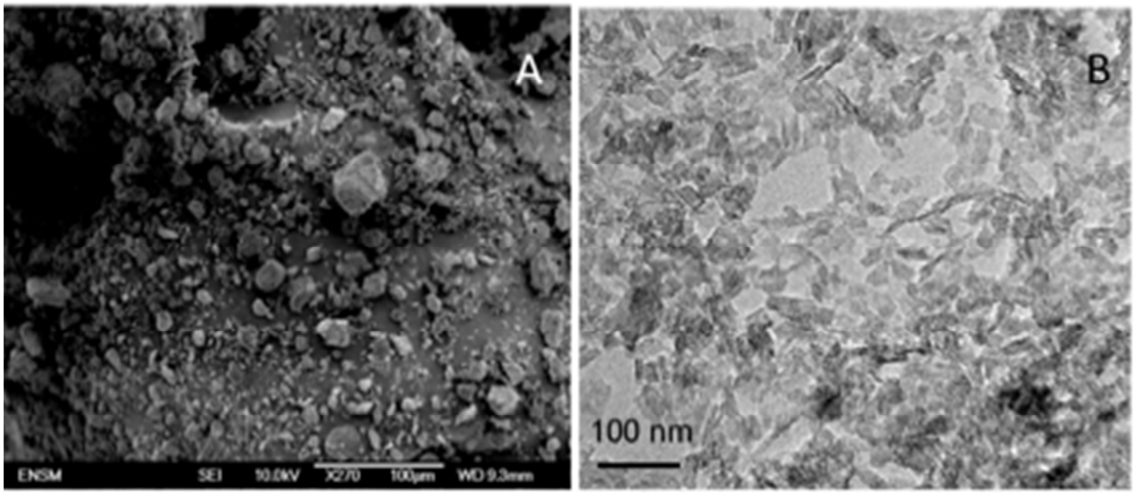


Fig. 2. Diffractogram of the boehmite NP.

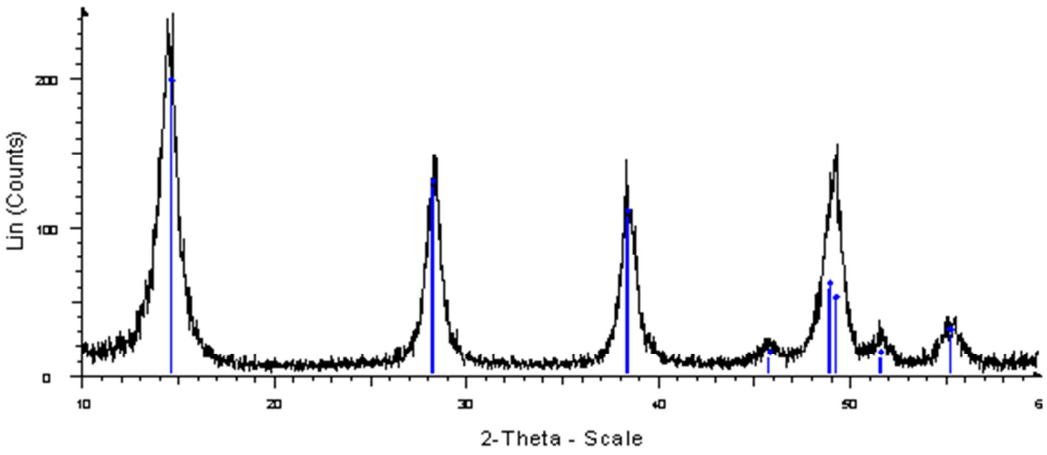


Fig. 3. Average diameter of particles depending on the pH in water and variation of zeta potential.

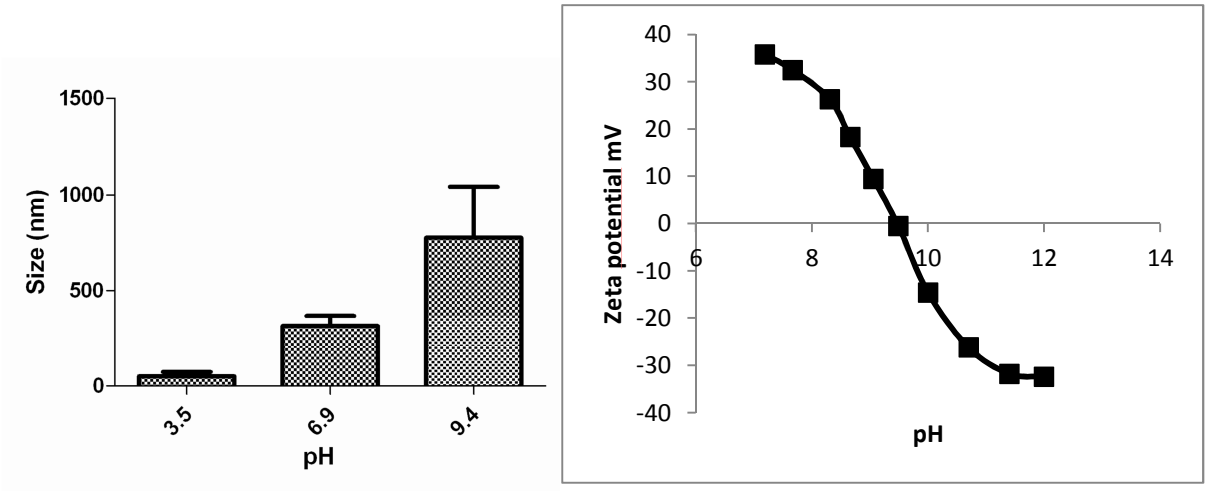
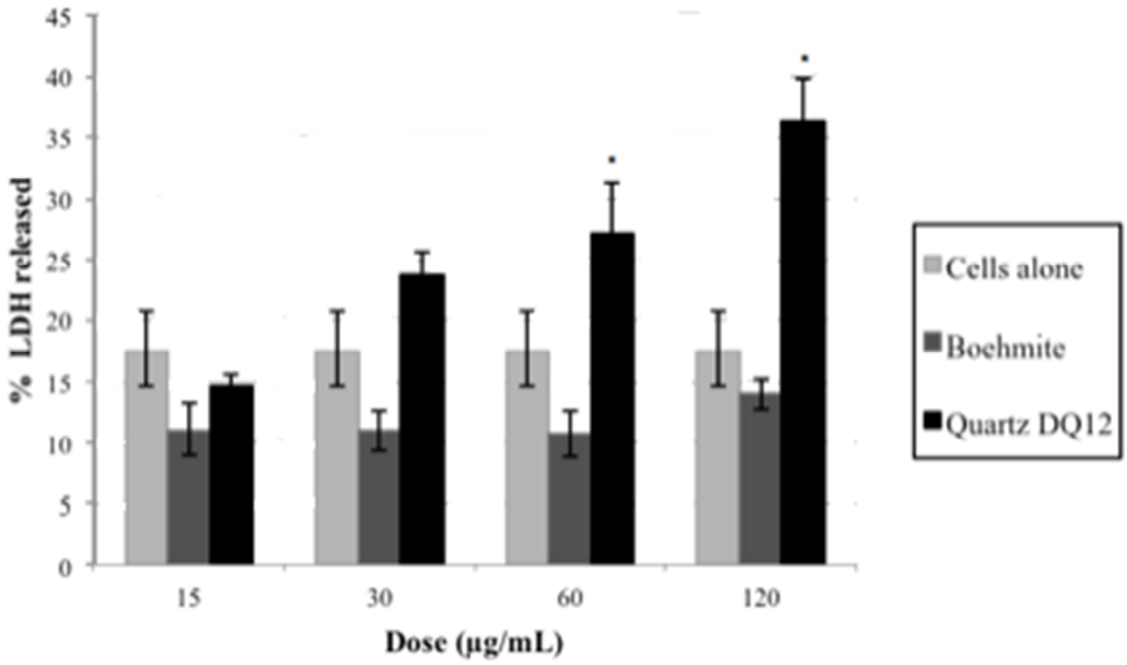


Fig. 4. Toxicity assessment. RAW 264.7 cells were incubated with boehmite NP for 24 h, and (A) cell damage measured by the amount of LDH released and (B).the TNF-α production (pg/ml) were assessed. This production was compared to that of cells alone (negative control of toxicity) and to that of cells incubated with DQ12 quartz (positive control) (n = 3, *p < 0.0001).

A.



B.

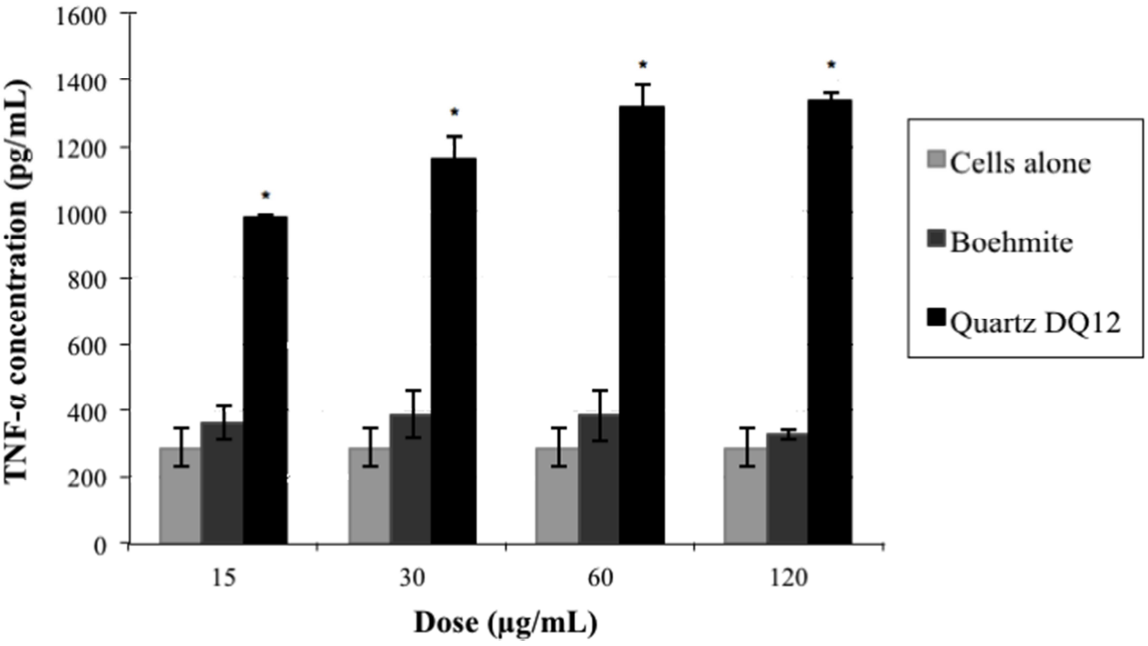


Fig. 5. Adsorption of TNF-α depending on the temperature (4 °C or 37 °C), the duration of incubation (3, 16, 24, and 48 h), the nature of the support (polystyrene or glass), and the presence of boehmite NP in cell-free conditions, TNF-α mass loss (Δm TNF-α).

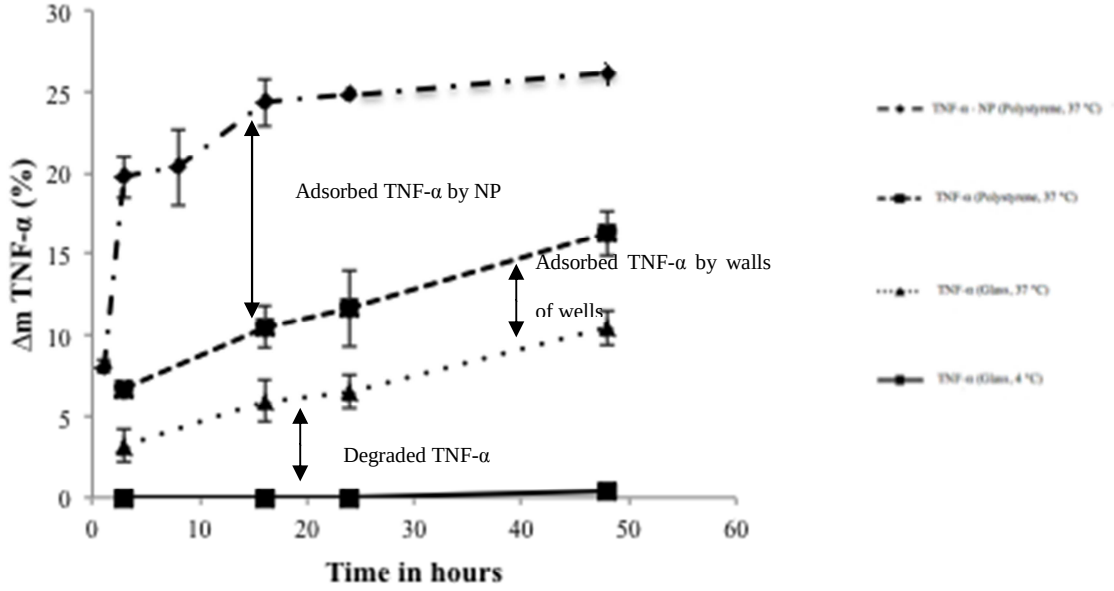


Fig. 6. Adsorption kinetics of different boehmite NP/TNF-α mass ratios (0.78, 1.56, 3.13, 6.25) for different times (1, 3, 16, and 24 h).

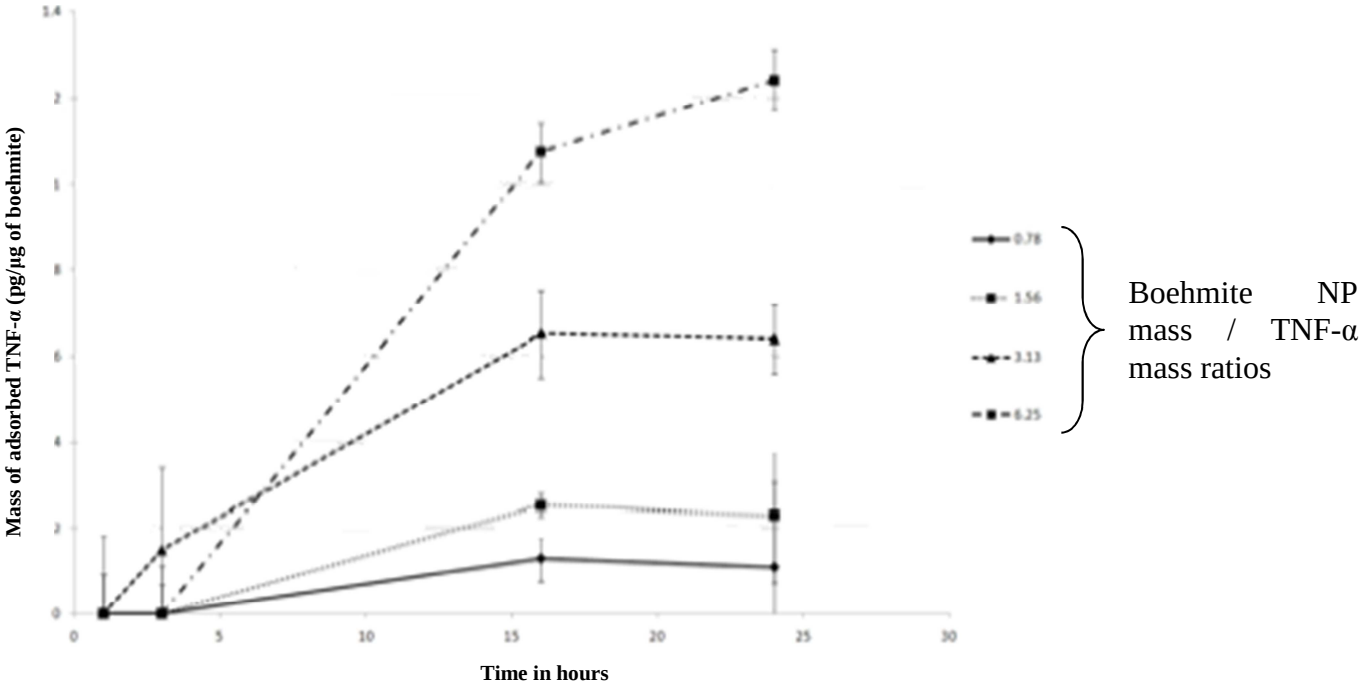


Fig. 7. Corrective curve representing the adsorbed TNF-α depending on the residual TNF-α after 24 h of incubation.

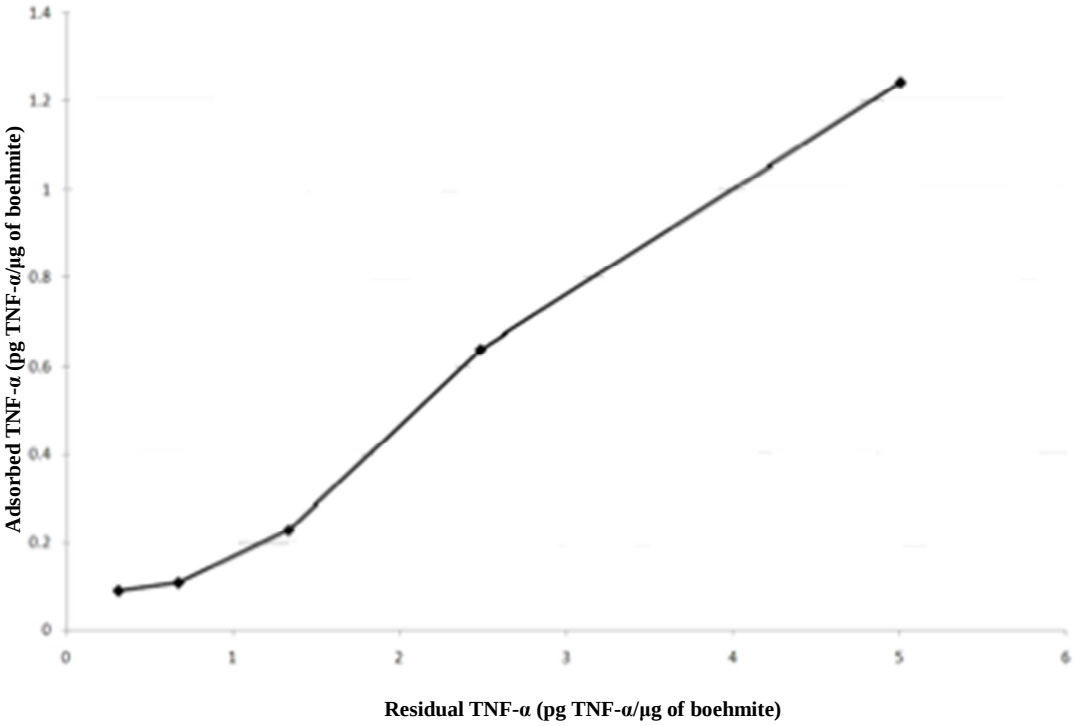
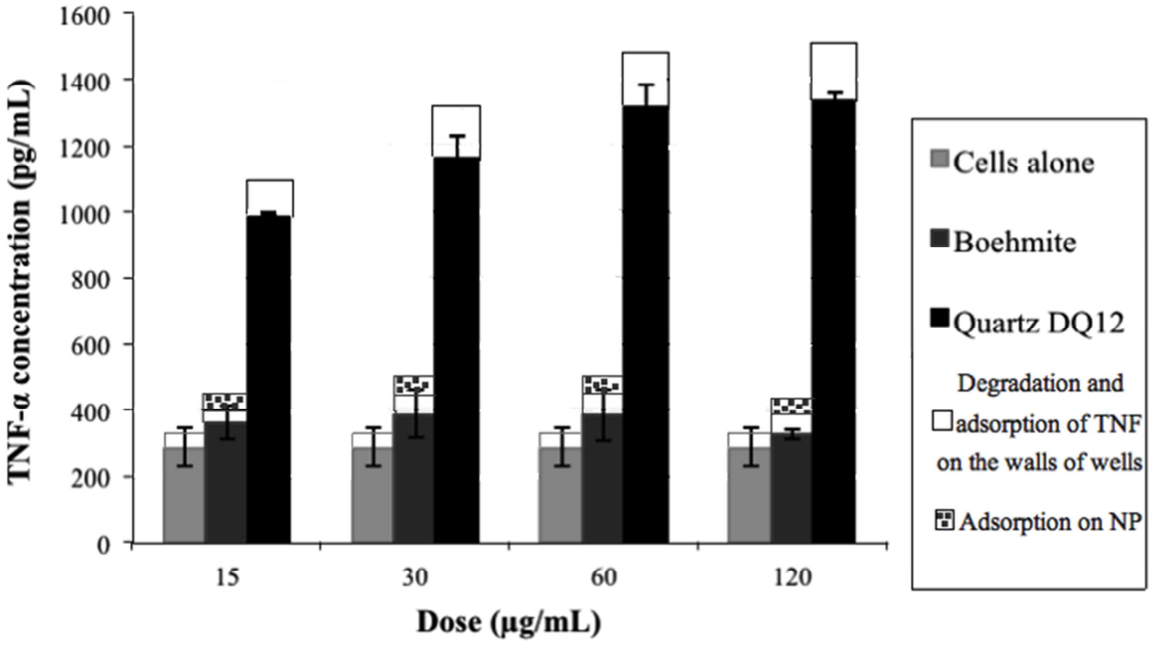


Fig. 8. Pro-inflammatory effect of boehmite NP as assessed by the TNF-α production (pg/ml) after correction of the adsorption and degradation artifacts.


TNF-α concentration (pg/mL)
1600
1400
1200
1000
800
600
400
200
0
15
30
60
120
Dose (µg/mL)
Cells alone
Boehmite
Quartz DQ12
Degradation and adsorption of TNF on the walls of wells
Adsorption on NP